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14. ABSTRACT We seek to design and implement a genetic circuit that based on multiple genetic markers is able to selectively recognize and destroy cancer cells, leaving healthy cells unaffected. In this project we focus on the MCF-7 breast adenocarcinoma cell line, a well-characterized cell line derived from a common form of breast cancer. MCF-7 cells overexpress Gata3, NPY1R and TFF1 mRNA relative to healthy cells. Based on our bioinformatics analysis, taking into account the three biomarkers allows for dramatically improved specificity in comparison to targeting single genes. We therefore design our circuit so that it only targets for destruction cells with high levels of mRNA of all three biomarkers (an AND gate). We have tested and successfully implemented each of the necessary circuit components: very efficient siRNA and microRNA gene knockdown, hBax dependent apoptosis, expression of mStaple (a short regulatory mRNA), and non-integrating lentivirus. Our current efforts are focused on careful characterization of the modules as a preliminary step for final implementation and fine-tuning of the full circuit. In the next step we will introduce all of the circuit components into HEK 293 and MCF-7 cells.					
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INTRODUCTION

Motivation and objective

We seek to design and implement a genetic circuit that based on multiple genetic markers is able to selectively recognize and destroy cancer cells, leaving healthy cells unaffected. In this project we focus on the MCF-7 breast adenocarcinoma cell line, a well-characterized cell line derived from a common form of breast cancer. MCF-7 cells overexpress Gata3, NPY1R and TFF1 mRNA relative to healthy cells. Based on our bioinformatics analysis, taking into account the three biomarkers allows for dramatically improved specificity in comparison to targeting single genes (Figure 1). We therefore design our circuit so that it only targets for destruction cells with high levels of mRNA of all three biomarkers (an AND gate).

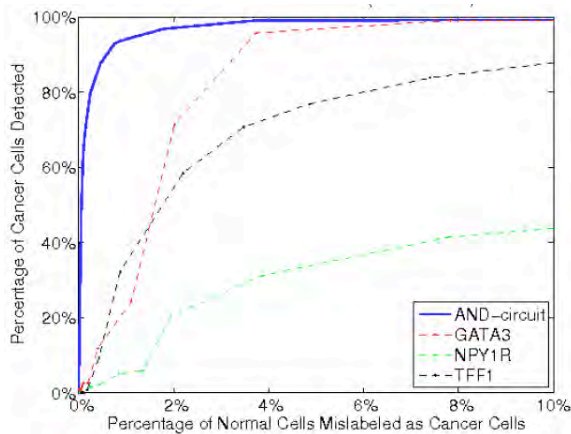


Figure 1: MCF7, breast adenocarcinoma cells overexpress Gata3, NPY1R and TFF1. Receiver operator characteristic (ROC) curves for single biomarkers and the AND-gate, visualize how taking into account three biomarkers improves specificity and selectivity.

BODY

I. Approach

We investigate two versions of the circuit design. The two approaches share many common components, but differ moderately in the way the cell state is being evaluated.

In approach I (Figure 1), three different shRNA are expressed, processed by Dicer to siRNAs and subsequently target the three chosen biomarkers of cancer (cell state evaluator module of the circuit). The target sites for the siRNAs are also artificially attached in the 3'UTR of the killer/reporter gene. When all the siRNA molecules are titrated away by the targeted mRNA molecules (high biomarker levels, cancer cells), the killer gene is expressed and leads to apoptosis. If any of the biomarkers is expressed at low level, the corresponding siRNA targets 3'UTR of the killer gene, resulting in cell survival. One of the potential problems in this approach is that the mRNA of biomarkers is targeted by siRNA also in non-cancerous cells and that may affect cells health. Also, there is no energetical preference for siRNA binding to the biomarker mRNA over the killer mRNA, and that may lower the killing efficiency even in cancer cells.

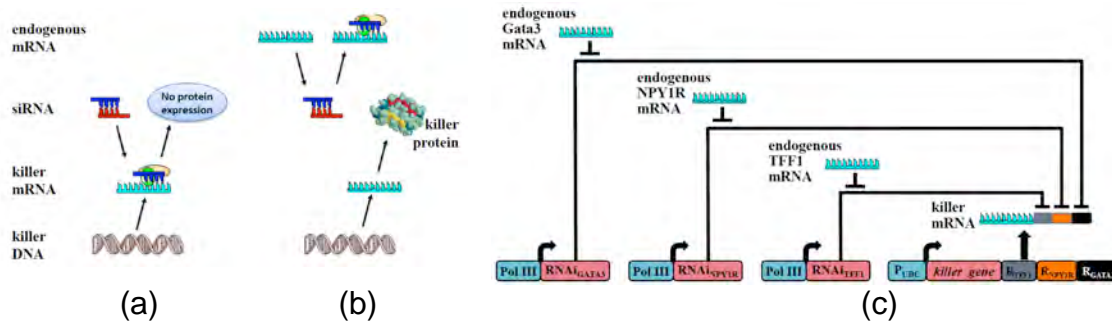


Figure 1: RNAi logic circuit-based approach I. (a-b) Killer protein (e.g. Bax) expression depends on levels of endogenous marker mRNA as mediated by siRNA interactions. (c) For the 3-input AND gate, the endogenous levels of Gata3, NPY1R and TFF1 all need to be high in order to titrate away the three engineered siRNAs and allow expression of the killer protein.

In the second approach (Figure 2), the components of our proposed circuit also include an apoptotic gene with an engineered regulatory sequence (RS), short interfering RNA (siRNA) directed against the RS, and a set of additional short mRNA sequences, mStaples. Each mStaple molecule is complementary to a specific cancer biomarker and partially complementary to a portion of the RS. The role of mStaple is to regulate siRNA mediated degradation of the apoptotic gene. In the absence

of mStaple, the RS forms a stem loop where the siRNA binding site is hidden and does not allow for siRNA binding and degradation of the mRNA. As a result, the cell undergoes apoptosis. When the mStaple binds to the RS, it enforces a conformational change of the sequence and exposes siRNA binding site. The mRNA of the apoptotic gene is degraded and the cell survives. The expected behavior is therefore abundance of the mStaple in normal cells and its shortage in cancer cells. In our system the mStaple is expressed similarly in all cell types, but its availability for binding of the RS depends on the level of endogenous genes – cancer biomarkers. The mStaple binds preferentially to the biomarker mRNA and with lower affinity to the RS. In normal cells with low biomarker levels some of the mStaple will be bound by the biomarker and some will target the RS to expose the siRNA binding sequence. In cancer cells, when the biomarker level is high, the mStaple will be titrated away, causing no disruption in expression of the apoptotic gene, and ultimately cell death.

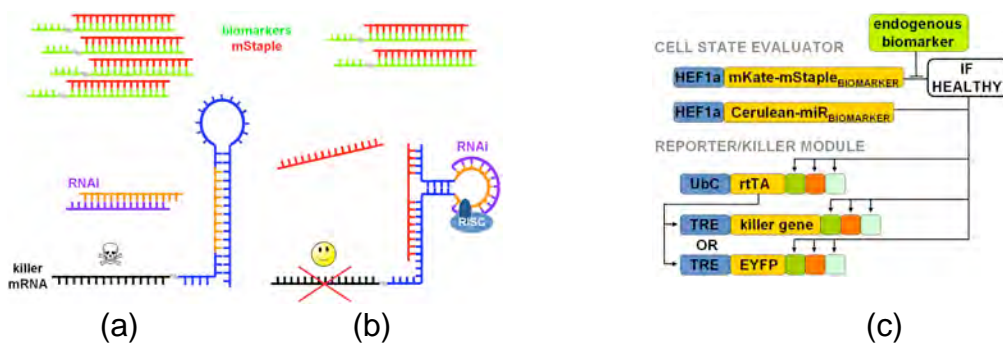


Figure 2: RNAi-based logic circuit, approach II. Similarly as in approach I, killer protein is dependent on the endogenous biomarker mRNA. For testing purposes, we replace the killer gene with a reporter, EYFP gene in the first implementation of the circuit.

Both circuit designs require efficient siRNA mediated knockdown and testing of the pro-apoptotic genes. Controllable expression of the short mStaple sequence is also needed for the second

approach. We will address progress on each of these modules in the next section.

II. Results

II.a. Circuit design and construction

We have build and tested multiple versions of the circuit design, and the optimal set of constructs is shown in Figure 3. The output of the biosensor is driven by and inducible promoter TRE (tetracycline response element). TRE promoter is active only in the presence of rtTA (reverse tetracycline-dependent transactivator) protein complexed with exogenously added doxycycline. Such design has two major advantages. First, it allows for keeping the output in the OFF state without administration of doxycycline, regardless of the biomarker levels in the cell. This provides additional safety level, ensuring that there is no uncontrolled production of the killer gene. Additionally, the regulatory sequence can also be placed in the 3'UTR of the activator, rtTA gene allowing for regulation at multiple levels. The transcription of the activator, as well as the microRNA, is driven by a strong and ubiquitous human Elongation Factor 1 α promoter (hEF1 α). The identity of the promoters driving expression of the Staple molecules will be further established to ensure titration of each mStaple by the corresponding biomarker.

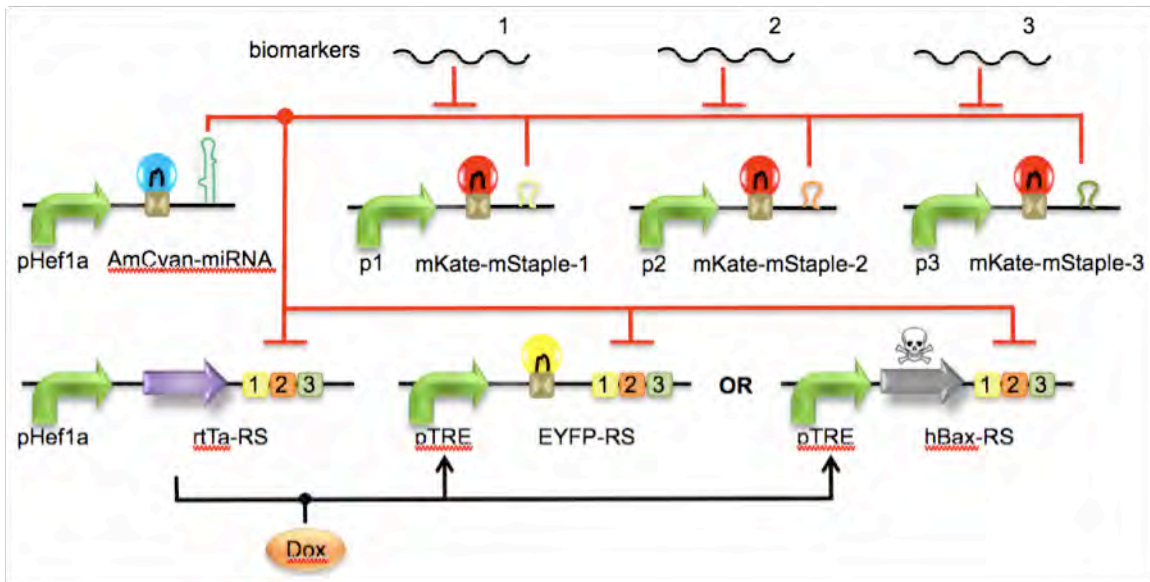


Figure 3: Current design of the AND logic circuit for detection and destruction of cells characterized by overexpression of three different biomarkers.

II.b. MicroRNA gene knockdown is dependent on the conformation of the 3'UTR regulatory sequence

We started the assembly of the 3-input AND gate with creating and testing a single biomarker sensor dependent on Gata3. The mStaple molecule binds to the Gata3 gene with perfect complementarity and it is only partially complementary to the designed regulatory sequence. Therefore, it will bind to the actuation gene 3'UTR with lower affinity, and preferentially only after all Gata3 binding sites have been satisfied. Upon mStaple binding, the RS mRNA fragment undergoes a conformational change,

exposing siRNA binding site. Such design allows for Dicer mediated degradation of the actuation mRNA in cells with low target mRNA (Gata3) levels. Figure 4 shows predicted structures of Gata3-dependent regulatory sequence alone (A) and in the presence of mStaple (B).

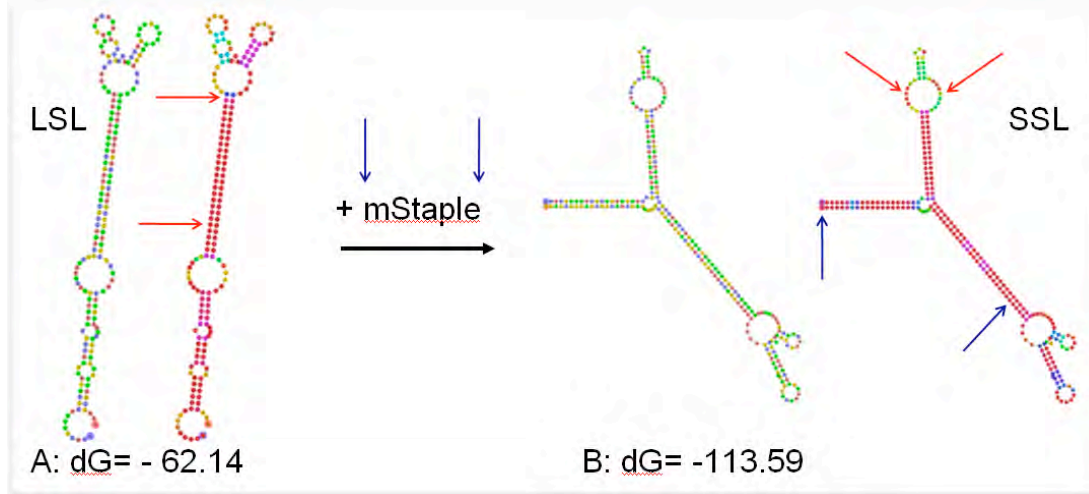


Figure 4: Conformational change of the 3'UTR Regulatory Sequence triggered by mStaple (blue arrows); A: In the absence of mStaple, the RS forms a long stem loop (LSL) hiding the siRNA binding site (red arrows) – the actuation gene will be expressed; B: in the presence of mStaple the RS changes conformation to form a short stem loop (SSL) with siRNA target site exposed – the actuation gene will be degraded. Secondary structure prediction was performed using mfold [1,2].

As established and reported previously, we use microRNA-FF4 and the corresponding FF4 target site (3), that provide the best knockdown efficiency. To verify that the designed regulatory sequence is capable of hiding and exposing the microRNA binding site we have tested EYFP-3'UTR knockdown, where the 3'UTR contained one of the following:

- (a) 4xFF4, 4 repeats of FF4 target sequence (maximal knockdown),
- (b) SSL-FF4, short stem loop (SSL) containing FF4 target site – fragment of RS simulating mStaple bound conformation (knockdown expected),
- (c) LSL-FF4, long stem loop (LSL) containing FF4 target site – the full regulatory sequence (knockdown not expected without mStaple present).

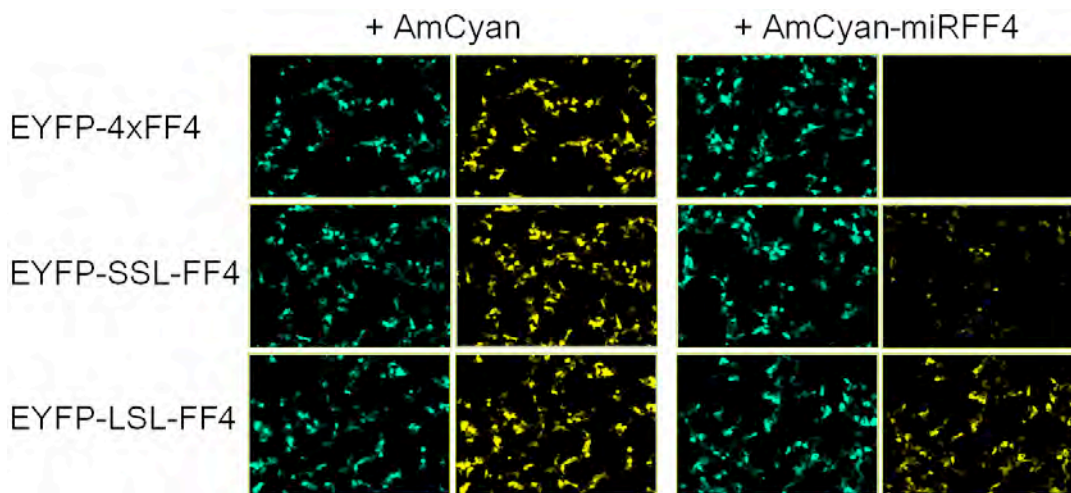


Figure 5: 293FT cells were co-transfected with 200ng of TRE-EYFP-3'UTR and 600 ng of AmCyan AmCyan-miR-FF4 expressing constructs. As expected, the conformation LSL prevents gene knockdown.

The results agree with our predictions very well. The full regulatory sequence hides siRNA target site, whereas the shorter sequence that can only form SSL and therefore simulates the presence of mStaple, exposes the target site (Figure 5). Although knockdown for the case of SSL-FF4 construct is not full, we can increase knockdown efficiency by placing multiple copies of the regulatory sequence in the 3'UTR (as shown in Figure 5 for EYFP-4xFF4 and in Figure 6).

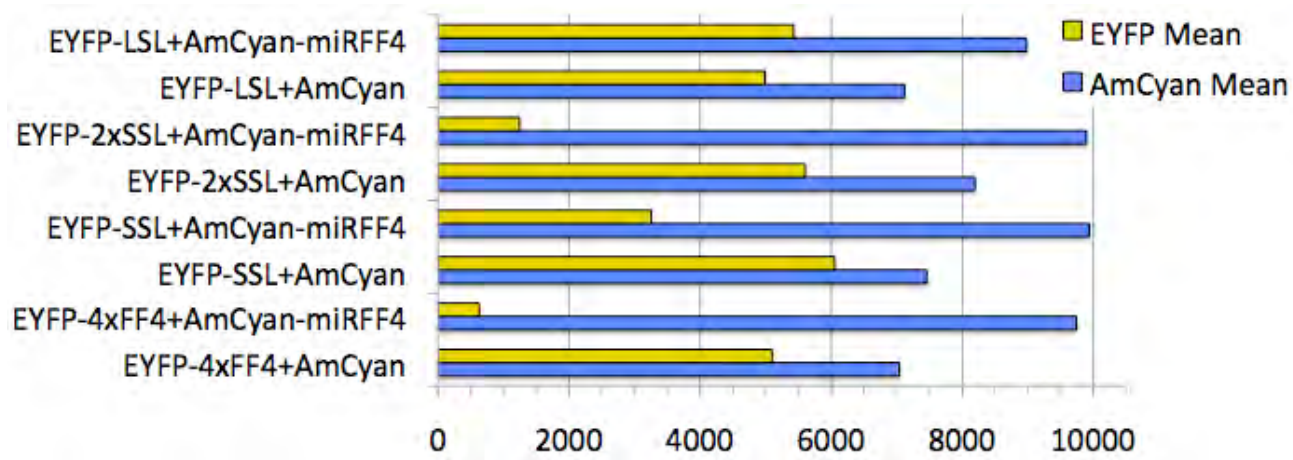


Figure 6: FACS results showing maximal knockdown in the case of EYFP-4xFF4 (AmCyan-microRNA versus AmCyan control); significant knockdown for EYFP-SSL, that is further improved by placing two copies of SSL in the 3'UTR: EYFP-2xSSL; and finally no knockdown in the case of EYFP-LSL construct.

II.c. Mstaple dependent gene knockdown

In the next step we have tested if we can induce conformational change of the regulatory sequence in vitro (Figure 7) and in vivo (Figure 8) by measuring microRNA knockdown efficiency with and without mStaple.

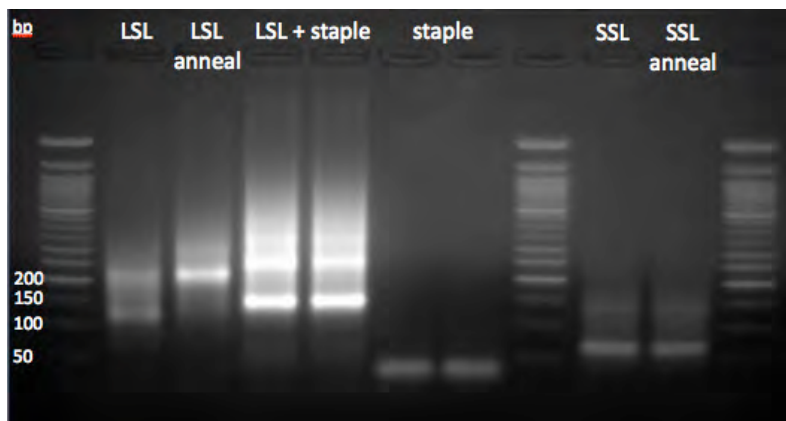


Figure 7: Gel image showing LSL, SSL and mStaple oligonucleotides. When LSL is subjected to an anneal protocol by itself, it forms one type of secondary structure (line 3). When the same LSL oligonucleotide is annealed in the presence of the corresponding staple molecule, it forms a different secondary structure (lines 4 and 5). Band corresponding to the staple molecule (lines 6 and 7) is missing from lines 4 and 5, confirming hybridization of LSL and staple.

As shown before, co-transfection of microRNA producing construct (AmCyan-miRFF4) and EYFP-SSL does not result in significant knockdown compared to the control (AmCyan +EYFP-LSL) since the conformation of the LSL prevents binding of the microRNA. When the same constructs are co-transfected with mStaple RNA oligonucleotide, the microRNA knockdown efficiency is increased, although the effect is not yet satisfactory. The single stranded RNA oligonucleotide may be highly

unstable in transfection media and in the cell, and that may be the primary reason for the small effect of the staple molecule. To verify that, we will subsequently test modified oligonucleotides, such as LNAs, that are characterized by comparable, or better DNA binding affinity and much longer half-life.

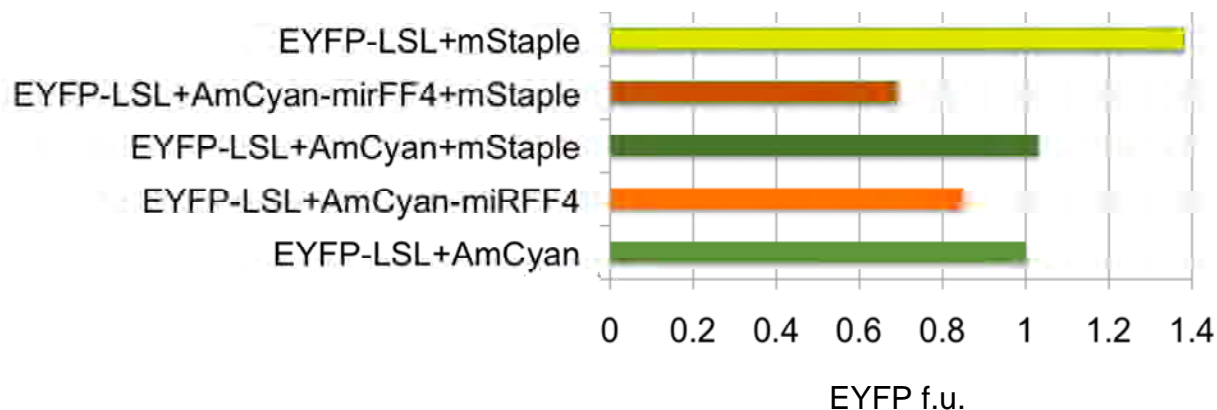


Figure 8: 293FT cells were co-transfected with 100ng TRE-EYFP-LSL and 300 ng of AmCyan AmCyan-miR-FF4 expressing constructs and 5pmol of mStaple RNA. Although presence of mStaple enhances gene knockdown, the effect is not yet satisfactory.

III. Outreach

The following students were involved in the project and mentored:

Genia Dubrovsky (junior project, 2009)

Anna Igorevna Podgornaia (rotation student, 2009)

Hattie Chung (Amgen scholar, 2010)

KEY RESEARCH ACCOMPLISHMENTS

- Design and theoretical prediction of a novel mRNA binding based mechanism for distinguishing cells in different states (manifested by different mRNA expression levels);
- Design and experimental verification of the 3'UTR regulatory sequences SSL and LSL that can expose or hide microRNA target site regulating gene knockdown;
- Demonstration of gene knockdown regulation by a short RNA oligonucleotide, mStaple.

REPORTABLE OUTCOMES

The work has been presented on the following conferences:

- The American Society for Cell Biology annual meeting, Philadelphia, PA, Dec 11-15, 2010
- SB5.0: The Fifth International Meeting on Synthetic Biology, Stanford, CA, Jun 14-17, 2011

Patents filed:

U.S. S/N: 12/587,994

Titled: Detection And Destruction Of Cancer Cells Using Programmed Genetic Vectors

CONCLUSION

We have predicted and demonstrated experimentally that microRNA dependent gene knockdown can be regulated by 3'UTR secondary structure of the target gene. The secondary structure can be changed with the help of exogenously added short RNA oligonucleotide, mStaple. Our current efforts are focused on increasing the dynamic range of the circuit response, that is optimizing the mStaple dependent gene knockdown. In the next step we will test the circuit components in HEK 293 and MCF-7 cells to demonstrate sensing of the endogenous Gata3 mRNA. We will also develop a computational model of the circuit to guide the optimization of the circuit components.

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APPENDICES